CROSSLINKING OF COLLAGEN*

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It has been shown that the introduction of a few stable crosslinks into the skin protein can greatly increase the resistance of the leather to deterioration by moist heat and perspiration.

A method based on stress–strain measurements has been developed for the determination of the number of crosslinks introduced into collagen, and a study made of the crosslinking potentialities of a number of bifunctional and polyfunctional compounds. Aldehydes appear to be most promising, cyanuric chloride and its derivatives also offer possibilities, while di-isocyanates are relatively inefficient due mainly to their low solubility in and sensitivity to water. Of the aldehydes, glutaraldehyde and acrolein were the most efficient both with respect to the number of crosslinks introduced and their stability.

Leathers tanned with glutaraldehyde show good resistance in moist heat and perspiration tests and pretannage or retannage of chrome leather with this aldehyde greatly improves its resistance.

Introduction

The work described in this paper forms part of a programme of research on the mechanism of the deterioration of leather and the development of more stable tannages.

One of the most common causes of deterioration is the combined action of heat and moisture as, for example, in the drying of wet leather at raised temperatures or during prolonged storage under warm, moist conditions. In wear also, the leather is exposed to warmth from the body and moisture in the form of perspiration. In the earlier stages of the work it was shown that damage was due to both degradation of the skin protein (collagen) and to changes in the tanning agent, mechanical failure eventually resulting under the various stresses applied to the leather in use. One aspect of the work, therefore, was to examine the changes occurring in the protein as a result of warm, moist storage and to consider ways of improving resistance under such conditions.

Breakdown of the collagen may take place in two ways, scission of the polypeptide chains or disorganisation of the molecular structure similar to that occurring in the conversion of collagen to gelatin. Both probably occur to some extent, the first facilitating the second.

Using fluorodinitrobenzene for the detection of α-amino-groups liberated, it has been shown that hydrolytic breakdown can be quite extensive in humid atmospheres at temperatures above 30°, especially if the pH is low. Loss of amino-acids and the production of volatile nitrogen suggest that another type of breakdown is also involved, possibly similar to that occurring when proteins are exposed to ultra-violet light or γ-radiation.[1,2] With untanned collagen, scission of the peptide chains is soon followed by disorganisation of the molecular structure and the protein becomes almost completely soluble in water. This is shown diagrammatically in Fig. 1. With tanned collagen the degree of breakdown appears to be of the same order but disorganisation of the molecular structure is much less and there is little increase in solubility.

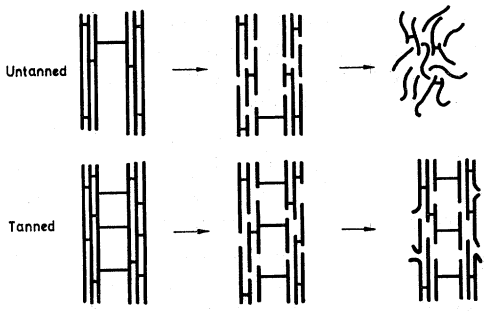

Fig. 1. *Diagrammatic representation of the breakdown of collagen and tanned collagen under warm, moist conditions*

The three-chain helical macromolecule of collagen (top left) has few stabilising intramolecular crosslinks and the number of intermolecular crosslinks is also low. Scission of the polypeptide chain can thus soon lead to disorganisation of the molecular structure. Introduction of crosslinks by tanning stabilises the structure and retards disorganisation

Vegetable tannins which rely mainly on multiple, weak hydrogen bonds for their binding to collagen are not very effective in stabilising the structure. Chromium salts which introduce crosslinks between carboxyl groups in the protein are very effective stabilising agents to moist heat alone. Unfortunately these bonds are not stable to perspiration, the lactate ions present in the latter competing with protein carboxyl groups for chromium and causing detannage.

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The effect of treating chrome leather with increasing concentrations of a synthetic perspiration followed by exposure to moist heat is illustrated in Fig. 2. As the concentration of perspiration was increased, the leather became progressively darker in colour, thinner, stiffer and more papery in feel. In extreme cases it was shrunken and could be broken quite readily by repeated flexing.[3]

As the work progressed it became increasingly evident that the presence of even a few stable crosslinks could greatly improve the resistance of leather to deterioration and a study of the crosslinking potentialities of a number of bifunctional and polyfunctional compounds was undertaken. These need not necessarily act as complete tanning materials in themselves, but could be combined with the more conventional tanning materials, thus extending the range of compounds likely to be economically acceptable.

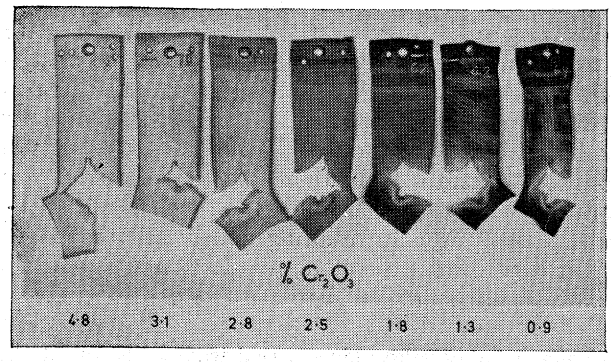

Fig. 2. *Chrome leathers after treatment in increasing concentrations of a synthetic perspiration containing sodium lactate followed by storage over water at* 40° *for* 13 *weeks*

Experimental

Determination of degree of crosslinking

The problems of crosslinking collagen differ in some respects from those involved with gelatin In the modification of gelatin the object is generally to introduce a few crosslinks, but to stop short at the point at which infinitely large molecules are formed and insoluble products result. This is really the point at which the crosslinking of collagen starts and, since the aim is to attain maximum stability, the more crosslinks introduced the better, within fairly wide limits. There are also stereochemical problems involved both with respect to the accessibility of the reactive groups to the crosslinking agent and their relative positions with respect to one another.

Thus the molecular dimensions of the crosslinking agent are important, it must be small enough to penetrate readily, if possible into the tropocollagen helix, and at the same time must be large enough to bridge the gap between the reactive groups of the protein.

The problem of assessing the degree of crosslinking of collagen is also rather more difficult than with gelatin. Shrinkage temperature gives some indication of the degree of crosslinking but it is a somewhat empirical measurement being affected by such factors as pH, rate of heating and, more particularly, the solvent. Witnauer & Fee[4] suggest that the decreased accessibility of the fibres to water which results from tannage, contributes to hydrothermal stability and it is possible that the shrinkage temperature in the absence of solvent or better still, the melting temperature as defined by Flory[5] would give a better indication of the degree of crosslinking. There are, however, a number of difficulties associated with such methods. Another possibility is to measure the swelling of the skin, but, once a few crosslinks are introduced, changes in water uptake are very small and difficult to measure experimentally.

A more promising approach appeared to be that of Wiederhorn and co-workers[6] based on the assumption that, on heat denaturation, collagen swells and forms a random elastic network to which the theories of rubber-like elasticity can be applied at temperatures above which the influence of hydrogen bonds become negligible.

From measurements of extension and application of the equation (below) developed by Flory[7,8] for an ideal rubber, the number of crosslinks per unit chain mass can be calculated.

$$f = \boldsymbol{R}T\rho V_2^{1/3}(\alpha - \alpha^{-2})/M_c$$

where f = force in dynes per unit cross-sectional area
$\boldsymbol{R}$ = gas constant
T = absolute temperature
ρ = dry density of the rubber-like material
V_2 = volume fraction, i.e. vol. dry sample/vol. wet sample
α = extension ratio, i.e., stretched length/original length
M_c = mean molecular weight per crosslinked chain segment.

The work of Wiederhorn and his co-workers[6] suggests that the equation can be applied satisfactorily to denatured kangaroo tail tendon (KTT) in the temperature range 21—65° and also for formaldehyde-tanned tendon at higher temperatures provided the extensions are less than 15% of the unstressed length. More recently, Flory & Spurr[9] have concluded that rat tail tendon crosslinked with *p*-benzoquinone, exhibits rubber-like elasticity.

A number of assumptions are made in the derivation of the equation, however, and it is not certain that the values obtained by this method represent the absolute number of crosslinks. Nevertheless, it seemed likely that it would serve as a useful measure of the relative degree of crosslinking attained.

Naidus & Browne[10] have applied the method to chrome-tanned tendon and Sykes[11] to tendon tanned with epoxides and difluorodinitrodiphenyl sulphone, with some success. In some of this earlier work misconceptions seem to have arisen in the interpretation of M_c and its relationship to the number of crosslinks per molecular weight unit. Reference back to the derivation of the above equation by Flory, however, made it clear that two crosslinked chain segments as defined by M_c must be involved in each crosslink and hence there is one crosslink per molecular weight unit $2M_c$.

Kangaroo or wallaby tail is by far the most suitable material for this type of work. It provides a relatively pure source of collagen of uniform structure and is strong enough to allow reasonably large extending forces to be applied. Long lengths of tendon can readily be dissected out from one tail so allowing a number of experiments to be carried out on comparable material and, if required, chemical analyses to be made also. Kangaroo and wallaby tails were supplied by the Zoological Society, London, but, in the main, tendons were obtained directly from Australia.

The apparatus used for the stress–strain measurements is shown in Fig. 3. The tendon is soaked back in water and then denatured in boiling water for 2 min. It is mounted between the two clamps A and B immersed in a beaker, enclosed in a larger thermostat and left to

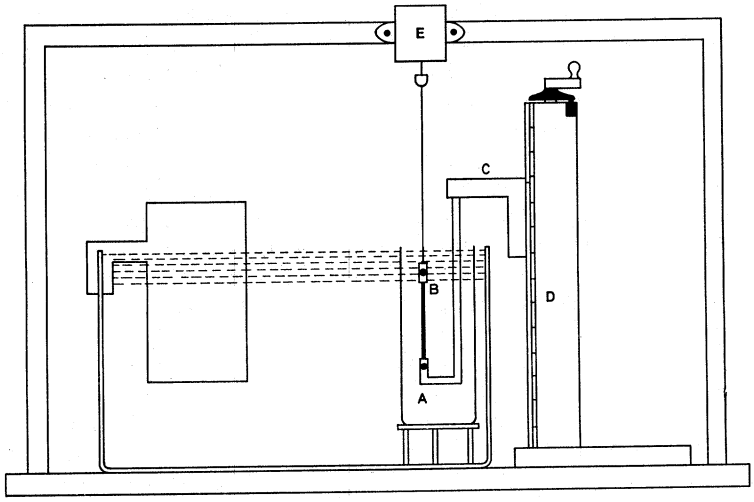

Fig. 3. *Stress–strain extensiometer*
A and B—clamps C—platform of stand D E—strain gauge transducer

equilibrate for 2 h. at the temperature of the test, usually 65°. The clamp A is rigidly attached to the platform C which can be raised or lowered by the rotation of a screw mounted in the stand D. A collar calibrated in millimetres fixed to the screw enables small vertical displacements of the order of 1 mm. to be measured. The other clamp B is attached to a strain gauge dynamometer type UF1 (Langham Thompson Ltd.) (E) used in conjunction with a J.L.T. transistorised strain gauge indicating type RML 6019. The tendon is extended in equal steps of about 0·2 cm. each to approximately 115% of its original length and the equilibrium tension recorded at each stage. It is then sliced off at each clamp, blotted, weighed, the wet volume determined by a specific gravity bottle method, and the tendon dried and reweighed. From these measurements and the unstressed length the cross-sectional area and the volume fraction can be calculated (for full details see Cater[12]).

With all tendons tested in this way a plot of f against $(\alpha - \alpha^{-2})$ approximated to a straight line passing through the origin indicating that the Flory equation is applicable. From the graph the gradient M_c can be calculated and from this the number of crosslinks per molecular weight unit

With the untanned tendons values of M_c varying between 375,000 and 190,000 were obtained corresponding to 0·4—0·8 crosslinks per molecular weight unit of 300,000, i.e. per tropocollagen macromolecule. The method measures only crosslinks in excess of those necessary to give a giant network in the first place.[8] On the basis of present ideas regarding collagen structure, a further three crosslinks would be required for this, two to crosslink the three chains of the helix and one to join the tropocollagen molecules together. The results, therefore, indicate values between 3·4 and 3·8 for the total number of crosslinks per tropocollagen unit. This is rather less than some workers have suggested on the basis of reactions with hydroxylamine and hydrazine[13] and more in line with values suggested by Bello[14] and Veis and his co-workers.[15]

The ages of the kangaroos from which the tendons were obtained were not known and hence it was not possible to relate the variations in M_c with age. Tendons which had been stored for several years gave rather lower values of M_c, of about 60,000, suggesting that, as generally believed, with acid-soluble collagen some crosslinking can occur *in vitro*.

All measurements on untanned tendon were on the limit of the method, the tendons tending to dissolve during the equilibration period and too much stress should not be laid on the values obtained; they do, however, appear to be of the right order.

Reaction of collagen with difluorodinitrodiphenyl sulphone

An attempt was made to assess the validity of the method by chemical means using *pp'*-difluoro-*mm'*-dinitrodiphenyl sulphone (FF sulphone), a reagent used by Zahn[16] for crosslinking collagen and wool. The products of the reaction of this compound with the ε-amino groups of lysine and hydroxylysine can be isolated after hydrolysis and the amounts of the bis-amino compounds found should give an indication of the number of crosslinks formed. The possibility that some of the bis-derivatives formed arise from reaction with amino groups in the same chain cannot, however, be excluded. The number of α-amino groups in collagen is very small and derivatives of these will be negligible compared with those involving ε-amino groups.

It was originally intended to isolate and determine the three bis-derivatives from the actual tendons used for the stress–strain measurement. Unfortunately, it was not possible to obtain samples of these from Zahn as hoped and the necessary time could not be devoted to preparation. It was, therefore, decided to rely on the data obtained by Zahn and his co-workers[16] for a collagen foil made from tendon. The number of crosslinks formed was calculated from their results on the assumption that no intra-chain crosslinks were formed and the values compared with results obtained by the stress–strain method (Table I). The results are quoted as numbers of crosslinks per molecular weight unit of 100,000, i.e., as moles of crosslink per 100,000 g. Values reported by Sykes[11a] some years ago are also quoted. These are higher than those obtained in the present investigation due to a different interpretation of M_c and should be divided by two for comparison with the other results. With the short reaction time the chemical estimate of crosslinks is higher than the physical, probably because the reaction with the tendon is incomplete owing to slower penetration of the reagent. With longer reaction times agreement is better. Zahn & Nischwitz[16b] consider that the lower value of 4·9 obtained by the isotopic method is due to a secondary breakdown of crosslinks to give unipoint fixation; presumably this would set in earlier with the more readily penetrated foil.

Table I

Chemical and physical estimates of crosslinks introduced by FF-sulphone

Method	Short reaction time		Long reaction time	
	h.	Crosslinks per 10^5 g.	h.	Crosslinks per 10^5 g.
Chemical—Zahn & Wegerle[16a]	—	—	120	6·2
Radio-chemical—Zahn & Nischwitz[16b]	12	6·2	100	4·9
Stress–strain—present investigation	16	2·6	120	6·0
Stress–strain—Sykes[11a]	4	4·0	144	12·1

On the whole, therefore, the results indicate that the stress–strain method gives a reasonably good estimate of crosslinking. Obviously a number of assumptions have to be made and there are difficulties involved in deciding on the correct values to be used for dry density of tanned and untanned tendons. However, even if the method does not give true values for the number of crosslinks, it should provide useful information on the relative crosslinking efficiency of different compounds and the best conditions for their use.

Crosslinking with aldehydes

Aldehydes, particularly formaldehyde are among the simplest bifunctional compounds which have been used for tanning. This group of compounds was, therefore, considered first.

Typical values for the number of crosslinks introduced per molecular weight unit of 100,000 are given in Table II, for three aldehydes. Excess aldehyde was used and the treatment was for 18 h. at room temperature. With all three aldehydes the number of crosslinks increases with pH; on the whole, glutaraldehyde is the most efficient, although acrolein is nearly as good. When lower concentrations of aldehyde (0·15 moles per 100 g.) were used, the superiority of glutaraldehyde was more marked, the corresponding values for numbers of crosslinks introduced at pH 8·0 being formaldehyde four, glutaraldehyde twelve, acrolein six and glyoxal nine.

Twelve seems to be the maximum number of crosslinks which can be introduced with glutaraldehyde. Increasing the reaction time to 48 h. or carrying out the reaction at 40° instead of 20° gave no increase. Assuming that it is the amino groups which are involved, this, in fact, approaches the maximum number possible, namely 15—16 per 100,000.

In a further experiment, tendons were tanned with a number of different aldehydes for 16 h. at room temperature. With formaldehyde, glyoxal, glutaraldehyde, acrolein and dialdehyde starch the pH of tannage was 8·0; owing to their instability, tannage with the other dialdehydes was carried out at lower pH values—6, 7 and 6·5 for malonic, succinic and adipic dialdehydes, respectively. (Fig. 4).

Again, glutaraldehyde and acrolein introduced the largest number of crosslinks, succinic dialdehyde and glyoxal introduced nearly as many, formaldehyde, malonic dialdehyde and dialdehyde starch about half as many and adipic dialdehyde only about two.

If the compound is to be useful in increasing the resistance of leather to deterioration, the stability as well as the number of crosslinks is important. Stability to moist heat is essential, and stability to acid is also desirable, since if used in conjunction with traditional tanning materials treatment in acid liquors will almost certainly be involved. For certain purposes stability to alkali is also necessary as, for example, in the case of boots for farm workers.

To test stability, the tendons were heated in boiling water for 7 h. or immersed in 2N-sulphuric acid for 24 h. at laboratory temperature. Since the number of crosslinks introduced

Table II

Effect of pH on crosslinking with aldehydes

	pH			
	4·0	5·0	6·5	8·0
Formaldehyde	—	4	5	6
Glutaraldehyde	6	7	8	10
Acrolein	5	—	6	11

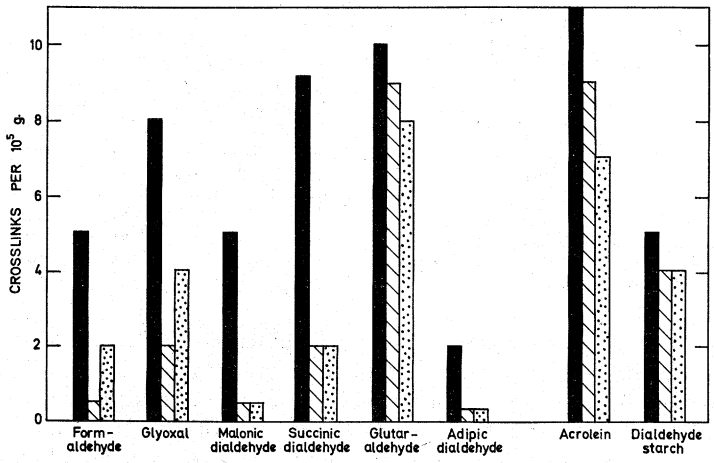

Fig. 4. *Crosslinking with aldehydes. Moles of crosslinks introduced per 10^5 g. and their stability*

■ Crosslinks introduced
/// „ stable to 7 h. in boiling water
⁝⁝⁝ „ „ „ 24 h. in N-H_2SO_4 at 20°

increases with pH, stability to at least moderate concentrations of alkali is almost certainly good. Results presented in Fig. 4 indicate that glutaraldehyde, acrolein and dialdehyde starch are clearly superior to the other aldehydes with respect to stability. Why this should be so is not clear; presumably the larger number of crosslinks introduced with glutaraldehyde is related to the chain length, but why the bonds once introduced should vary in stability from those formed with the other dialdehydes is not obvious. With formaldehyde a different mechanism is involved, reaction occurring first with amino groups followed by secondary condensation with amide and guanidino groups[17] and it is probably the bond involved in this latter condensation which is labile.

A difficulty of much of this work has been the determination of the amount of aldehyde fixed. Even with formaldehyde it is not always certain that all the aldehyde is recovered by distillation from acid, and with glutaraldehyde this is certainly not so.

A few experiments have been carried out with ^{14}C-labelled glutaraldehyde synthesised from adipic acid and then determining the amount of aldehyde bound by scintillation counting. The results obtained, together with the number of crosslinks introduced are given in Table III.

Table III

Reaction of glutaraldehyde with tendon collagen

pH of tannage	Aldehyde bound	Crosslinks	Amino groups	
			Free	Reacted
	Moles per 10^5 g.			
Untanned	0	0·2	30·8	—
pH 4·0	21	4·9	14·7	16·1
pH 5·0	24	7·5	11·1	19·7
pH 6·5	32	8·0	8·2	22·6
pH 8·0	38	8·3	8·0	22·8

The tendons were also analysed for lysine and hydroxylysine in the hope that losses of these amino-acids might indicate the extent of the reaction with the ε-amino groups. Even if the bonds formed between glutaraldehyde and these groups are not stable to hydrolysis, it is possible that, as with ε-dinitrophenyl-lysine,[18] breakdown does not give free lysine. The amounts of the two basic amino-acids recovered did, in fact, decrease with the pH of tannage, the decreases running roughly parallel with the amounts of aldehyde bound. However, until it is possible to isolate the glutaraldehyde–lysine derivatives or their breakdown products, it is not certain that there is a direct relationship and that there are, in fact, some unreacted ε-amino groups remaining after tannage at pH 8·0. In other experiments the recovery of free

lysine has also approximated to similar limiting values and preliminary attempts at the formol titration have indicated a similar proportion of unreacted lysine.

There was only a small loss of arginine, and it seems unlikely that the guanidino group will react with glutaraldehyde to any appreciable extent below pH 9·0. The amounts of glutaraldehyde bound, however, exceed a 1:2 ratio with ε-amino groups and, if it is assumed that two such groups are involved in each crosslink, it follows that there must be two or more molecules of glutaraldehyde associated with many of the amino groups. Presumably the aldehyde polymerises in some way.

Crosslinking with di-isocyanates

Attempts have been made to crosslink collagen with di-isocyanates. Their low solubility in and sensitivity to water has presented difficulties, the compounds tending to polymerise within and on the fibres rather than to combine with the protein.

Reaction with hexamethylene di-isocyanate and toluene di-isocyanate dissolved in pyridine or in benzene containing a tertiary amine, led to an apparent decrease in M_c. There was, however, considerable polymerisation, increases in weight of up to 100% being observed and it seems doubtful whether the denaturation prior to stress–strain measurements was satisfactory.

In attempts to reduce polymerisation while, at the same time, permitting crosslinking to occur, consideration was given to the preparation of soluble addition compounds, impregnating the collagen with these and then decomposing them under conditions favourable to reaction with the amino groups. Succinimide and bisulphite addition products of both hexamethylene and toluene di-isocyanates were prepared. With the succinimide compounds, seven and eleven crosslinks respectively were introduced and with the bisulphite compounds three to four.

Crosslinking with cyanuric chloride derivatives

Cyanuric chloride contains three reactive chlorine atoms and derivatives in which one or two of these are replaced by a dye molecule form the basis of the successful range of reactive dyes, e.g. the Procions of Imperial Chemical Industries Ltd. Cyanuric chloride itself has a very low solubility in water but with 50% aqueous acetone it was possible to introduce up to seven moles of crosslinks per 10^5 g. of collagen (Table IV).

Table IV

Crosslinking with cyanuric chloride and its derivatives

Compound	pH of treatment	Crosslinks per 10^5 g.
Unsubstituted cyanuric chloride	8	6
	9	7
	10	6
Methoxy derivative	8	6
	9	5
Amino derivative (hydrochloride)	7	4
	8	7
	9	5
Sulphonate derivative (Na salt)	7	4
	8	5
5-sulphonaphth-1-ylamino derivative (Na salt)	7	4
	8	3
	9	1
	10	0·5
Bis-44′-diaminostilbene-22′-disulphonic acid derivative (Na salt)	7	6
	8	4
	9	1

The reactivity of the three chlorine atoms in cyanuric chloride is not the same, after one has reacted the second and third are less reactive, depending on the nature of the substituent. The introduction of a methoxy group is reported to increase the reactivity of the two remaining chlorines[19] and certainly with this compound it was possible to introduce nearly as many crosslinks as with the cyanuric chloride itself. A similar number were also introduced by the amino

compound, but with the sulphonate and the larger sulphonaphthylamino derivative the numbers were fewer.

With all these compounds, therefore, the third chlorine atom retains some reactivity. Optimum crosslinking occurs at about pH 7·0—8·0 and no advantage appears to be gained by raising the pH further. Presumably the increased reactivity of the amino groups at the higher pH is more than counterbalanced by interaction of the third chlorine atom with water. Rather surprisingly, the stilbene with two reactive triazine residues was no more effective than the other compounds.

The stability of the crosslinks introduced by some of the compounds is shown in Fig. 5. With the simpler compounds stability to hot water is relatively good, but acid stability is rather poor. Included in Fig. 5 are results with tetrakis(hydroxymethyl)phosphonium chloride, a compound which has been suggested as a tanning material in conjunction with resorcinol.[20]

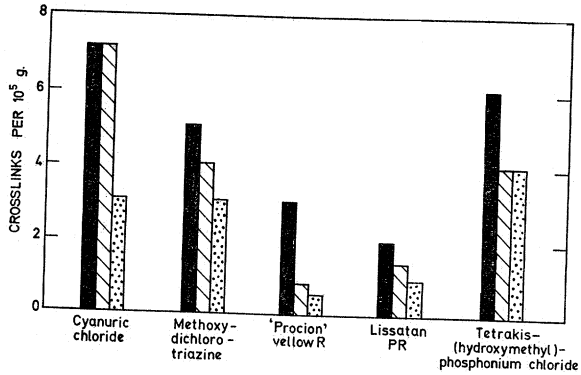

Fig. 5. *Crosslinking with cyanuric chloride derivatives. Moles of crosslinks introduced per* 10^5 *g. and their stability*

■ crosslinks introduced
/// „ stable to 1 h. in boiling water
⁝⁝⁝ „ „ „ 16 h. in N-H_2SO_4 at 20°

Conclusions

Of the compounds tested, the aldehydes appear to be the most promising both with respect to numbers of crosslinks introduced and their stability. Cyanuric chloride derivatives are also relatively efficient, but in view of the low activity of the last chlorine atom it seems likely that these compounds will prove more useful for the introduction of other compounds into collagen as with the Procion dyes, than as crosslinking agents in themselves.

Of the aldehydes, glutaraldehyde and acrolein are by far the most efficient crosslinking agents. Acrolein, however, is not very pleasant to use and high concentrations are required to give the best results. Trials carried out with glutaraldehyde both here and in the United States have shown that it can be used as a tanning material in itself to give a washable leather which is very resistant to perspiration and when used in conjunction with chromium salts greatly improves the stability of the resulting leather. This increased stability is illustrated in Fig. 6 which shows the effect of successive treatments with a synthetic perspiration on glutaraldehyde, chrome and glutaraldehyde–chrome combination tanned leathers. Samples of the leather were extracted up to six times with a synthetic perspiration, each extraction being for 4 or 5 days at 40°. The chrome leather showed signs of damage after only one extraction and after the sixth, it dried out to a hard and horny product. The glutaraldehyde-tanned leather, on the other hand, showed little damage even after six extractions, and although it became rather thinner, it still remained relatively soft. The leather tanned first with glutaraldehyde and then chrome, was rather more affected but was still reasonably flexible and soft after six extractions.

It is only fair to say that Filachione and workers at the Eastern Regional Laboratories of the U.S. Dept. of Agriculture have been carrying out tannery trials with glutaraldehyde over the past few years[21] and, while they have developed the practical side, the present work demonstrates the reasons underlying its advantages.

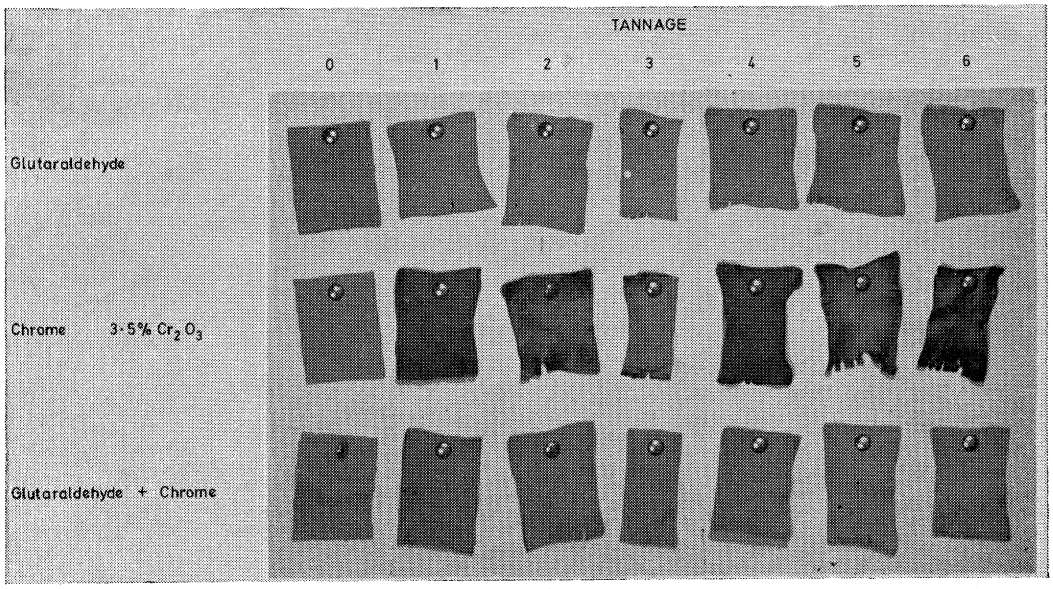

Fig. 6. *Glutaraldehyde and chrome leathers after successive treatment with synthetic perspiration*
(each treatment for 4 days at 40° using 20 ml. of solution per g. leather)
Synthetic perspiration: 2% sodium lactate, 2% sodium chloride, 0·1% glycine, 16 ml. 0·1N-$NaHCO_3$ per 100 ml.

Acknowledgments

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British Leather Manufacturers' Research Association,
Milton Park,
Egham, Surrey

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